

# Inositol 1,4,5-trisphosphate receptor subtype 3 in pancreatic islet cell secretory granules revisited

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**ABSTRACT** It has been reported that the inositol 1,4,5-trisphosphate receptor subtype 3 is expressed in islet cells and is localized to both insulin and somatostatin granules [Blondel, O., Moody, M. M., Depaoli, A. M., Sharp, A. H., Ross, C. A., Swift, H. & Bell, G. I. (1994) *Proc. Natl. Acad. Sci. USA* 91, 7777–7781]. This subcellular localization was based on electron microscope immunocytochemistry using antibodies (affinity-purified polyclonal antiserum AB3) directed to a 15-residue peptide of rat inositol trisphosphate receptor subtype 3. We now show that these antibodies cross-react with rat, but not human, insulin. Accordingly, the anti-inositol trisphosphate receptor subtype 3 (AB3) antibodies label electron dense cores of mature (insulin-rich) granules of rat pancreatic  $\beta$  cells, and rat granule labeling was blocked by preabsorption of the AB3 antibodies with rat insulin. The immunostaining of immature, Golgi-associated proinsulin-rich granules with AB3 antibodies was very weak, indicating that cross-reactivity is limited to the hormone and not its precursor. Also, the AB3 antibodies labeled pure rat insulin crystals grown *in vitro* but failed to stain crystals grown from pure human insulin. By immunoprecipitation, the antibodies similarly displayed a higher affinity for rat than for human insulin. We could not confirm the labeling of somatostatin granules using AB3 antibodies.

Inositol 1,4,5-trisphosphate ( $IP_3$ ) acts via specific receptors present on the membrane of target organelles to mobilize  $Ca^{2+}$  stores (1). Several subtypes of  $IP_3$  receptor have now been cloned (2–4). Following the demonstration that receptor subtype 3 is expressed in pancreatic islets (5), immunolocalization studies indicated that  $IP_3$  receptor subtype 3 ( $IP_3R$ -3) is present in secretory granules of the  $\beta$  cell (6, 7). A possible direct effect of  $IP_3$  on mobilization of  $Ca^{2+}$  from granule stores was proposed (6–8).  $IP_3R$ -3 was also found in somatostatin-containing granules of the  $\delta$  cell (6).

Although a model involving  $IP_3$ -stimulated  $Ca^{2+}$  mobilization from granules might seem attractive [and has been proposed for bovine adrenal medullary secretory vesicles (9)], it is not supported by direct studies on the mobilization of  $Ca^{2+}$  from  $\beta$ -cell organelles (10, 11). Furthermore, the apparent localization of  $IP_3R$ -3 to the electron-dense core of  $\beta$ -cell granules (6, 7) does not fit that expected of a transmembrane protein, and it is difficult to ascribe this discrepancy solely to sectioning artefacts showing tangential views of the granule membrane or to perturbation of granule ultrastructure during tissue preparation, as suggested (6). Since the 15-residue sequence of  $IP_3R$ -3 used for immunization (residues 2656–2670) constitutes the C-terminal cytoplasmic region of the receptor (2, 5, 12), an alternative explanation (6) of the granule core localization of  $IP_3R$ -3 immunoreactive sites being due to receptor degradation and sequestration of the epitope into the inner space of the granule appears untenable. We thus sus-

pected a possible cross-reactivity of the polyclonal anti  $IP_3R$ -3 with insulin itself (or with some other component of the core of secretory granules) and set out to investigate this possibility by high-resolution immunocytochemistry. Our results indicate that the  $IP_3R$ -3 antibodies (AB3) do indeed cross-react with insulin but not proinsulin and that there is no direct evidence at the ultrastructural level for localization of the  $IP_3R$ -3 to secretory granules of either insulin ( $\beta$ ) or somatostatin ( $\delta$ ) cells.

## MATERIALS AND METHODS

**Tissue Processing.** Adult rats were fixed by vascular perfusion with 1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4); pancreas fragments were processed for immunocytochemistry by low temperature embedding in Lowicryl K4M (13) and thin sectioning or direct cryoultramicrotomy according to Tokuyasu (14). In addition, pancreatic islets isolated by the collagenase method (15) as well as cultures of AtT20 cells were fixed and processed as indicated above.

**Insulin Crystallization.** Rat insulin (from Novo-Nordisk, Gentofte, Denmark) or human insulin (from Eli Lilly) was crystallized as described (16) using 1 mg of insulin in each case. The crystals were fixed in 1% glutaraldehyde in phosphate buffer at pH 6.5 and directly processed for cryoultramicrotomy.

**Transfected AtT20 Cells.** AtT20 (transformed pituitary corticotroph) cells [clone D16, a generous gift of Armen Tashjian (Harvard School of Public Health, Boston)] were grown in culture and stably transfected with the rat insulin II gene as described (17). Control (nontransfected) and transfected cells were fixed and processed for cryoultramicrotomy.

**Electron Microscope Immunocytochemistry.** Ultrathin cryosections and sections of Lowicryl-embedded tissue were collected on nickel grids and immunolabeled with the anti- $IP_3R$ -3 antibodies by the protein A-gold method (gold size, 10 nm). For double labeling of the same sections, the anti- $IP_3R$ -3 antibodies were detected with goat anti-rabbit IgG coupled to 6-nm gold particles; mouse monoclonal anti-insulin antibodies were detected with goat anti-mouse IgG coupled to 10-nm gold particles. As a control for specificity of insulin labeling, anti- $IP_3R$ -3 antibodies (25  $\mu$ g/ml) were preabsorbed with rat or human insulin (5–100  $\mu$ g/ml in 0.1 M phosphate buffer, pH 7.4/0.5% bovine serum albumin) or, as an additional control, with 10–500  $\mu$ g of the immunizing  $IP_3R$ -3 peptide per ml, for 2 h at room temperature or overnight at 4°C.

**Immunoprecipitation.** Mono-A14- $^{125}I$ -labeled rat insulin II was custom labeled to a specific activity of 380  $\mu$ Ci/ $\mu$ g (1 Ci = 37 GBq) by Anawa Biomedical Services, Wangen, Switzerland. Mono-A14- $^{125}I$ -labeled human insulin (specific activity, 325  $\mu$ Ci/ $\mu$ g) was the gift of Hoechst. Each tube for immuno-

precipitation contained 3 nCi of labeled insulin and 1.25  $\mu\text{g}$  of affinity-purified anti-rat IP<sub>3</sub>R-3 in a final vol of 50  $\mu\text{l}$  of 0.2 M glycine/0.5% bovine serum albumin (RIA grade from Sigma), pH 8.8. After 20 h of incubation at room temperature, the tubes were chilled on ice. To each tube was added 50  $\mu\text{l}$  of ice-cold human IgG (3.6 mg/ml in 0.2 M glycine, pH 8.8) followed by 100  $\mu\text{l}$  of ice-cold polyethylene glycol (25% PEG 6000 in 0.2 M glycine, pH 8.8). After Vortex mixing, the tubes were centrifuged in the cold for 20 min at  $3000 \times g$ . The supernatant (free insulin) was decanted and the pellet was washed once with ice-cold 12.5% PEG. Radioactivity in the pooled supernatants and the pellets was then measured using a  $\gamma$ -counter. As a control for maximum binding, 5  $\mu\text{l}$  of crude guinea pig anti-porcine insulin antiserum (Sigma) was used in place of the anti-rat IP<sub>3</sub>R-3 antibodies. Nonspecific binding was estimated by omitting antibody. To determine whether unlabeled insulin could compete with the tracer, 1  $\mu\text{g}$  of rat (a mixture of rat insulins I and II) or human insulin was added to the tubes during the 20-h incubation.

**Antibodies.** Affinity-purified rabbit anti-rat IP<sub>3</sub>R-3 antiserum AB3 directed against the 15-residue C terminus of rat IP<sub>3</sub>R-3 (6, 7) (gift of Graeme Bell, Howard Hughes Medical Institute, Chicago) was diluted to a final concentration of 25–50  $\mu\text{g}/\text{ml}$ ; affinity-purified AP45 generated against a unique portion of the mouse IP<sub>3</sub>R-3 located between two of the transmembrane regions (gift of Alan Sharp, Department of Psychiatry and Behavioral Sciences, Johns Hopkins Medical Institutions, Baltimore) was used at a final concentration of 20–70  $\mu\text{g}/\text{ml}$ ; mouse monoclonal anti-insulin antibody (Mab3) was prepared and used as described (18, 19); goat anti-rabbit IgG coupled to fluorescein isothiocyanate was from Biosys (Compiègne, France); goat anti-rabbit IgG coupled to 6-nm gold particles and goat anti-mouse IgG coupled to 10-nm gold particles were from Anawa.

## RESULTS

**Anti-IP<sub>3</sub>R-3 Labels Mature, Insulin-Rich Secretory Granules in Rat Pancreatic  $\beta$  Cells.** Exposure of cryosections of rat islets to the AB3 (anti-IP<sub>3</sub>R-3) antibodies resulted in intense labeling of the electron-dense core of insulin-rich mature granules (Fig. 1A), with virtually no labeling of newly formed proinsulin-rich granules (data not shown). On mature granules, immunogold particles did not associate with the halo surrounding the densely labeled core or with the granule membrane (Fig. 1A). A similar distribution of the labeling was observed on sections of islets embedded at low temperature in Lowicryl K4M (data not shown). The distinct mature granule core labeling was abolished by preabsorption of the AB3 antibodies with rat insulin (Fig. 1B and Table 1). By contrast, preabsorption with human insulin or with immunizing peptide used to generate the AB3 anti-IP<sub>3</sub>R-3 antibodies failed to block the granule core labeling regardless of the concentration of peptide used for preabsorption (Table 1). No other cytoplasmic compartment of the rat  $\beta$  cell was labeled by these IP<sub>3</sub>R-3 antibodies. In contrast to a published report (6), no labeling of insulin granules was observed using the AP45 anti-IP<sub>3</sub>R-3 antibodies despite repeated attempts using a range of antibody concentrations (data not shown).

**Anti-IP<sub>3</sub>R-3 Labels Sections of Rat but Not Human Insulin Crystals.** To confirm our suspicion that the dense core labeling of rat mature secretory granules was due to insulin, we immunolabeled crystals of rat or human insulin grown *in vitro*. While cryosections of both species of insulin crystals were labeled by the anti-insulin monoclonal antibody (Fig. 1D and E), only the rat crystals stained with the AB3 anti-IP<sub>3</sub>R-3 antibodies (Fig. 1C).

**Anti-IP<sub>3</sub>R-3 Immunoprecipitates Radiolabeled Insulin.** To confirm that the AB3 anti-IP<sub>3</sub>R-3 antibodies truly cross-reacted with insulin, they were used to immunoprecipitate rat

Table 1. Labeling density (number of protein A-gold particles per  $\mu\text{m}^2$ ) over granular cores of rat islet  $\beta$  cells using affinity-purified AB3 anti-IP<sub>3</sub>R-3 antibodies (25  $\mu\text{g}/\text{ml}$ )

Preabsorption conditions	Labeling density	No. of granules
Control	134 $\pm$ 7	116
Rat insulin		
(5 $\mu\text{g}/\text{ml}$ )	78 $\pm$ 9	39
(20 $\mu\text{g}/\text{ml}$ )	47 $\pm$ 6	72
(100 $\mu\text{g}/\text{ml}$ )	15 $\pm$ 3	46
Human insulin		
(5 $\mu\text{g}/\text{ml}$ )	138 $\pm$ 8	62
(20 $\mu\text{g}/\text{ml}$ )	144 $\pm$ 8	130
(100 $\mu\text{g}/\text{ml}$ )	132 $\pm$ 8	121
IP <sub>3</sub> R-3 peptide* (500 $\mu\text{g}/\text{ml}$ )	161 $\pm$ 16	40

Preabsorption was overnight at 4°C. Data are means  $\pm$  SEM for the number of granules indicated.

\*C-terminal peptide of IP<sub>3</sub>R-3 was used for immunization to obtain the AB3 polyclonal antiserum. Note that, in other experiments, the peptide was used at concentrations ranging from 10 to 500  $\mu\text{g}/\text{ml}$  and no inhibition of immunolabeling of granules was observed at any concentration (data not shown).

or human <sup>125</sup>I-insulin. Using the rat insulin II tracer, 28% of label was specifically precipitated, whereas only 12% was precipitable using the human insulin tracer. When using the rat insulin II-labeled insulin, 100% of specifically bound radioactivity could be displaced by 1  $\mu\text{g}$  of unlabeled rat insulin, whereas this same amount of unlabeled human insulin displaced only 40% of the bound rat tracer. Taken together, these results show that the AB3 anti-IP<sub>3</sub>R-3 antibodies do indeed cross-react with insulin and display a higher apparent affinity for rat than for human insulin. Note, however, that the binding of labeled insulin was poor compared with anti-insulin antiserum, which was found to precipitate >90% of radioactivity regardless of the tracer (rat or human insulin) used. It is therefore concluded that the affinity of the AB3 anti-IP<sub>3</sub>R-3 antibodies is very low even for rat insulin.

**Double-Labeling of Rat  $\beta$  Cells with Anti-Insulin and Anti-IP<sub>3</sub>R-3 Antibodies Reveals Concordant Staining of Granules.** To confirm the apparent specificity of labeling of insulin by the AB3 anti-IP<sub>3</sub>R-3 antibodies, we performed a double-labeling experiment using a monoclonal (mouse) antibody specific for insulin and the polyclonal (rabbit) AB3 anti-IP<sub>3</sub>R-3 antibodies. The two types of antibodies were visualized on the same section by tagging with gold particles of different size; this revealed a complete overlap of the antigenic sites in mature (insulin-rich) granules with very little labeling by either antibody of proinsulin-rich granules (data not shown). This result confirms that an epitope present in the rat insulin molecule, but apparently masked or inappropriately presented in its precursor proinsulin, is the site of cross-reactivity with the AB3 anti-IP<sub>3</sub>R-3 antibodies.

**Anti-IP<sub>3</sub>R-3 Antibodies Do Not Label Somatostatin Granules.** It has been claimed that AB3 anti-IP<sub>3</sub>R-3 antibodies label somatostatin granules (6). In the present study, no such labeling by anti-IP<sub>3</sub>R-3 of somatostatin ( $\delta$ ) cell components was found using either immunofluorescence or the immunogold technique (data not shown). We attribute this discrepancy to the misidentification of  $\beta$  cells as  $\delta$  cells in the previous study (6).

**Labeling of AtT20 Cell Granules by Anti-IP<sub>3</sub>R-3 Is Dependent on the Presence of Insulin.** As a confirmation that AB3 anti-IP<sub>3</sub>R-3 antibodies cross-react with rat insulin in a cellular setting other than the  $\beta$  cell, we labeled control (nontransfected) or insulin-expressing (transfected) pituitary corticotroph AtT20 cells. Only the transfected cells, expressing high levels of rat insulin II, were stained with the IP<sub>3</sub>R-3 antibodies,

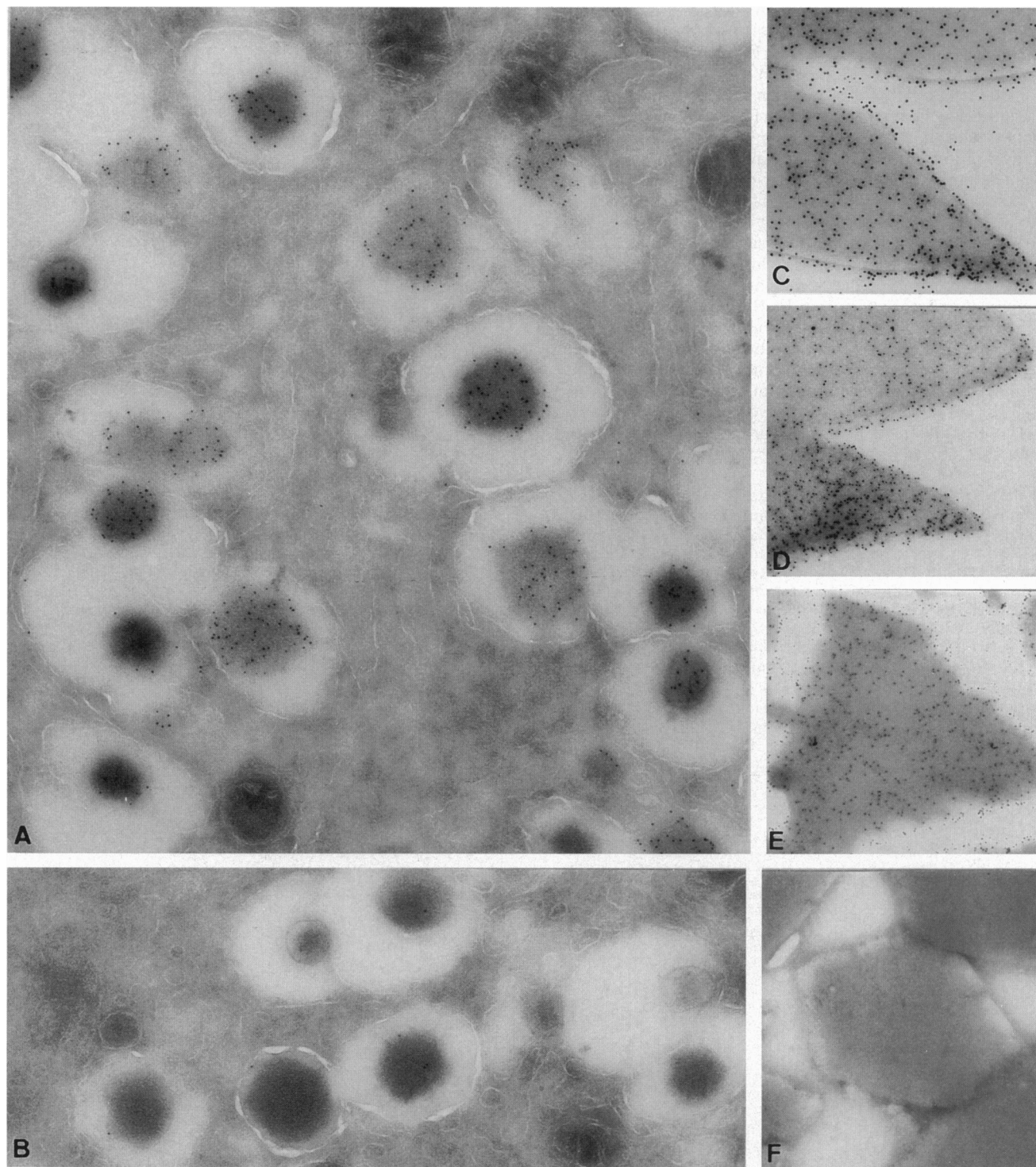


FIG. 1. (A) Ultrathin cryosection of a rat insulin ( $\beta$ ) cell immunolabeled with AB3 anti-IP<sub>3</sub>R-3 antibodies detected by the protein A-gold technique. Gold particles are limited to the dense granule cores. (B) Staining as in A but with anti-IP<sub>3</sub>R-3 preabsorbed with rat insulin (100  $\mu$ g/ml). (C–F) Cryosections of crystals of rat (C and D) or human (E and F) insulin immunolabeled with AB3 anti-IP<sub>3</sub>R-3 antibodies (C and F) or insulin monoclonal antibodies (D and E) detected by the protein A-gold technique. Cryosections of both species of insulin crystals were labeled by the anti-insulin monoclonal antibody (D and E), while the rat (C) but not the human (F) crystals stained with the AB3 anti-IP<sub>3</sub>R-3 antibodies. Anti-IP<sub>3</sub>R-3 antiserum AB3 was affinity purified and used at a final concentration of 25–50  $\mu$ g/ml. (A and B,  $\times 42,000$ ; C–F,  $\times 34,000$ .)

and the staining was restricted to the dense cores of secretory granules (data not shown).

## DISCUSSION

Both the postulate that IP<sub>3</sub>R-3 is present in insulin granules and more specifically the cores of such granules (6, 7) as well

as a model for the physiological role of IP<sub>3</sub>R-3 based on the postulate (8) have been the subject of recent controversy (20, 21). The morphological basis for this postulate depended on the use of polyclonal anti-IP<sub>3</sub>R-3 antisera. We were led to retest IP<sub>3</sub>R-3 distribution using these sera not because we supposed that IP<sub>3</sub>R-3 and insulin share common epitopes but because we were struck by the close topological resemblance

between IP<sub>3</sub>R-3 labeling and our previous extensive data on insulin labeling with both monoclonal and polyclonal antibodies (19, 22). The present data clearly indicate that the AB3 IP<sub>3</sub>R-3 (but not the AP45) antiserum cross-reacts with insulin, thereby accounting for this topological resemblance.

Immunocytochemistry is a powerful tool for studying cellular and subcellular localization of proteins. Assuming that all technical pitfalls of labeling are avoided, the results will ultimately depend on the specificity of the antibody used for the molecule being localized. In this particular instance, the quite unexpected cross-reactivity for insulin of the AB3 anti-IP<sub>3</sub>R-3 renders it inappropriate for subcellular localization of this particular receptor molecule in insulin-containing cells. It follows that previous studies localizing IP<sub>3</sub>R-3 to insulin granule cores using the AB3 anti-IP<sub>3</sub>R-3 are uninterpretable. Furthermore, and for unknown reasons, we have not been able to reproduce the previous observation (6) of immunolabeling of insulin granules using the AP45 anti-IP<sub>3</sub>R-3 antibodies. The immunocytochemical counterpart and subcellular localization of this receptor identified in islet cells by Western blotting (5–7) or postulated on functional grounds to be at work in insulin cells (10, 11) remain to be explored.

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